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# Experimental Gram-Negative Bacterial Sepsis: Reevaluation of the Ability of Rough Mutant Antisera to Protect Mice[1] (40231)

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During the past decade, several groups of investigators have reported that antisera from horses or rabbits immunized with various rough mutants of *Enterobacteriaceae* could passively transfer broad spectrum protection to mice against parenteral challenge with smooth strains of *Enterobacteriaceae*. This was attributed to "unmasking" of common core antigens in the mutants, allowing production of high titers of cross-reacting protective antibodies (1–3). However, a recent study by Ng and coworkers, while confirming the appearance of cross-reactive antibodies, failed to confirm any protective activity of rough mutant antisera in mice (4). Since the potential importance of antibodies that can confer broad spectrum protection against gram-negative sepsis is great, and since attempts are ongoing in our laboratory to identify chemical or serologic reagents that can prevent gram-negative septic mortality no longer preventable by antibiotics alone, we have re-examined the protective activity of antisera to several rough mutants of *Enterobacteriaceae*.

*Materials and methods. Infecting agents.* Three smooth species of *Enterobacteriaceae* were used to evaluate the broad spectrum protective activity conferred by antisera to rough mutant *Enterobacteriaceae: Escherichia coli* 018, isolated from the urine of a patient with acute pyelonephritis; *Proteus mirabilis*, also isolated from the urine of a patient with acute pyelonephritis; and the Caroli strain of *Klebsiella pneumoniae*, type II, obtained from Louis Chedid of the Pasteur Institute and kindly provided to us by William McCabe, Boston University Medical Center.

*Antiserum preparation.* Rabbit antisera were prepared against each of the above smooth challenge strains and against the following rough *Enterobacteriaceae* mutants: J5 mutant of *E. coli* 0111, chemotype Rc, kindly supplied by Edward Heath, University of Iowa; *Salmonella typhimurium* SL 1032, chemotype Rd, kindly supplied by Lawrence Rothfield, University of Connecticut; and *Salmonella minnesota* 595, chemotype Re, kindly supplied by Siegfried Schlecht, Max Planck Institut fur Immunbiologie, Freiburg. All bacterial suspensions for immunization were prepared by overnight culture at 37° in trypticase soy broth, concentrated by centrifugation, washed 3 times in sterile physiologic saline, and boiled for 10 min. The heat-killed bacteria were suspended in physiologic saline at concentrations of $10^8$/ml. Antiserum was prepared by injecting groups of three to six rabbits iv with 1.0 ml of the killed bacterial suspension for 3 consecutive days of each week for 2 weeks and bleeding 7 days after the sixth dose of antigen. These antisera were stored at −20° until use. For control purposes, normal rabbit serum was obtained from the same donors 5 to 7 days prior to immunization. The donors, 2–3 kg healthy albino New Zealand rabbits with no known previous illness, not previously injected with any agent and raised on antibiotic-free feed, were bled 10 ml each for these pre-immunization serum samples. The pre-immune sera were stored at −20° until use and then pooled in the same proportions as the post-immune sera. Postimmunization antibody titers to each of the rough mutant preparations, determined by a micro-agglutination technique,[2] ranged between 1:2560 and 1:10240

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[2] 0.1 ml aliquots of serial dilutions of sera in physiologic saline, starting at 1:10, were mixed with 0.1 ml of $1 \times 10^8$/ml suspensions of organisms previously washed 3× in physiologic saline; the mixtures were agitated 30 min on a Boerner type rotating machine at approximately 120 rotations per min at room temperature and agglutination read at 100 × magnification. Known negative and positive serum controls were always run concurrently.



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to the respective mutant. Preimmunization sera possessed no detectable agglutination titers against the Rd and Re mutants (<1:10) but in a few instances, specified in the Results section, 1:10 titers were detected to the Rc mutant or to an Ra chemotype (*S. minnesota* R60) obtained from Siegfried Schlecht. Antisera to the Re chemotype of *S. minnesota* 595 were also titered by an indirect hemagglutination technique, wherein purified lipopolysaccharide extracted from this mutant by phenol–chloroform–ether was used to sensitize erythrocytes (2). A titer of 1:320 was obtained with each anti-Re sera tested, compared to undetectable pre-immunization titers (<1:10).

*Experimental infections.* Outbred Swiss albino mice of mixed sexes, 20–25 g, were housed 10 per cage and fed antibiotic-free Purina Lab Chow during an acclimatization period of 5–7 days. Immediately before each study, each colony of 10 animals was randomly divided into test and control groups and injected iv with 0.25 ml antiserum, preimmune serum, or sterile pyrogen-free physiologic saline. One hour later all animals were challenged with *E. coli* 018, *P. mirabilis,* or *K. pneumoniae* suspensions grown overnight in trypticase soy broth at 37°. The organisms had been harvested by centrifugation, washed with physiologic sterile saline at room temperature, and suspended in saline at concentrations determined turbidimetrically at 580 nm. For ip challenge, 1.0 ml suspensions were injected, and 0.25 ml for iv challenge. Control and test animals were always challenged with the bacterial suspensions in an alternate manner to minimize any possible effects of changing bacterial numbers and viability during the total injection period. Mortality rates following *E. coli* and *P. mirabilis* challenge were taken at 96 hr. Mortality after *K. pneumoniae* challenge was recorded daily for 5 days.

*Results. Mortality dose-response relationships in untreated mice.* Mortality dose-responses following *E. coli* 018, *P. mirabilis,* and *K. pneumoniae* challenge are given in Fig. 1. Within the sensitive dose-response ranges, ip challenge consistently produced higher mortality rates than the iv route. With either route, mortality from *E. coli* and *P. mirabilis* increased minimally after 48 hr and no further deaths occurred after 96 hr. In contrast, *K. pneumoniae* proved lethal regardless of inoculum size, the latter determining only the rate of mortality as reported previously by McCabe (2). For all three challenge species, heat-killed suspensions administered iv or ip in inocula up to $5 \times 10^8$ produced no mortality, suggesting that bacterial replication was required for lethality when inocula of LD 95-100 or less were employed.

*Effect of pretreatment with rabbit antisera.* The ability of pooled rabbit antisera against the Rd chemotype of *S. typhimurium* SL 1032 and the Re chemotype of *S. minnesota* 595 to protect mice against challenge with *E. coli* 018 is given in Table I. Although protection was afforded by both antisera when compared with saline treated or untreated controls, preimmune sera from the corresponding donors was just as protective. This was true regardless of whether the sera were injected

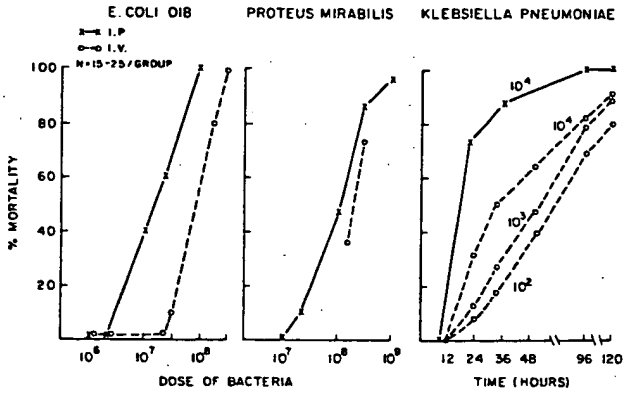


FIG. 1. Dose-response relationships for *E. coli* 018, *P. mirabilis,* and *K. pneumoniae,* type II, mortality in Swiss mice. *E. coli* and *P. mirabilis* mortality recorded at 96 hr.

TABLE I. EFFECT OF PRETREATMENT OF SWISS MICE WITH RABBIT ANTISERUM ON MORTALITY FROM *E. coli* 018.[a]

| iv Pretreatment (0.25 ml) | E. coli $1 \times 10^7$ ip (N) | % Mortality | E. coli $5 \times 10^7$ ip (N) | % Mortality | E. coli $1.5 \times 10^8$ iv (N) | % Mortality |
|---|---|---|---|---|---|---|
| None | (31) | 61 | (31) | 100 | (20) | 60 |
| Saline | (41) | 61 | (24) | 92 | | |
| *S. typhimurium* SL 1032 (Rd) antiserum | (13) | 23 | (17) | 71 | | |
| Preimmune serum | (28) | 21 | (30) | 77 | | |
| S. minnesota 595 (Re) antiserum | | | | | (20) | 20 |
| Preimmune serum | | | | | (20) | 15 |
| *E. coli* 018 antiserum | (15) | 0 | (10) | 20 | (20) | 0 |
| Preimmune serum | (31) | 19 | | | (20) | 20 |
| **ip Pretreatment (0.25 ml)** | | | | | | |
| Saline | | | (25) | 92 | | |
| *S. minnesota* 595 (Re) antiserum[b] | | | (26) | 46 | | |
| Preimmune serum[b] | | | (25) | 20 | | |
| *E. coli* 018 antiserum[b] | | | (24) | 0 | | |

[a] Each vertical column gives results from one separate study.
[b] Heated to 56°, 30 min.

iv or ip or whether *E. coli* challenge was performed by the iv or ip route. Despite its protective activity, the preimmune sera possessed no detectable (<1:10) agglutinating antibody to the *E. coli* 018 or to Ra, Rc, Rd, or Re rough mutants. In contrast to the anti-Rd and Re sera, specific antiserum to the infecting *E. coli* 018, possessing an *E. coli* 018 agglutinating titer of 1:5120, was significantly more protective than the corresponding preimmune serum ($P < 0.05$ for each of the two comparative trials).

Since pretreatment of mice with normal pre-immune rabbit sera protected against *E. coli* 018 sepsis, this activity was examined in more detail, Table II. In three separate trials, each comparing five different normal sera concomitantly with an untreated control group, marked individual serum variability was observed, and heating to 56° for 30 min did not destroy protective activity. Despite such protective activity, agglutinating antibody to *E. coli* 018 or to Rd or Re chemotypes of the rough mutants employed in these studies were undetectable (<1:10) in any normal serum. Two sera (C and E) possessed anti-Ra and Rc titers of 1:10. These sera provided less protection than others possessing no detectable titers.

Table III demonstrates that i.v. pretreatment of mice with rabbit antisera to the Rd and Re chemotypes of *Salmonella,* and to the Rc chemotype of *E. coli,* protected against iv (but not ip) challenge with *P. mirabilis.* However, preimmune sera from the corresponding donors provided comparable protection. None of the pre-immune normal sera possessed detectable agglutinating antibodies to the *P. mirabilis* or to Ra, Rc, Rd, or Re mutants employed in the present studies (<1:10). In contrast to the anti-mutant sera, iv pretreatment with specific antiserum to the infecting *P. mirabilis* provided significantly greater protection than the corresponding pre-immune serum against both iv and ip challenge ($P < 0.01$ and $P < 0.001$ respectively). This specific antiserum possessed an agglutination titer of 1:2580 to *P. mirabilis.*

Figure 2 demonstrates the ability of pooled

TABLE II. EFFECT OF PRETREATMENT OF SWISS MICE WITH SERUM FROM INDIVIDUAL NORMAL RABBITS ON MORTALITY FROM *E. coli* 018.

| iv Pretreatment (0.25 ml) | Individual donor | *E. coli* ($1 \times 10^7$ ip) (N) | % Mortality |
|---|---|---|---|
| Trial 1 | | | |
| Unheated serum | None | (10) | 95 |
| | A | (10) | 10 |
| | B | (10) | 50 |
| | C | (10) | 90 |
| | D | (10) | 100 |
| | E | (10) | 70 |
| Trial 2 | | | |
| Unheated serum | None | (20) | 60 |
| | F | (10) | 40 |
| | G | (8) | Immediate deaths from serum per se |
| | H | (9) | 0 |
| | I | (10) | 0 |
| | J | (10) | 0 |
| Trial 3 | | | |
| Heated serum (56°, 30 min) | None | (11) | 55 |
| | K | (10) | 0 |
| | L | (10) | 20 |
| | M | (10) | 0 |
| | N | (10) | 30 |
| | O | (9) | 0 |

rabbit antisera to the Rc chemotype of *E. coli* J5 mutant to protect mice against iv *K. pneumoniae* challenge. Pretreatment with the antisera retarded mortality, but the preimmune sera, possessing no detectable anti-*K. pneumoniae* or Rc antibodies (<1:10) provided equal protection. Heat-inactivation of antiserum at 56° for 30 min (end panel) did not alter the results.

Figure 3 demonstrates the ability of pooled rabbit antiserum to the Rd chemotype of *S. typhimurium* SL 1032 mutant to protect mice against iv *K. pneumoniae* challenge. Pretreatment with the antiserum retarded mortality when compared with saline treated controls, but the pre-immune serum, possessing no detectable anti-*K. pneumoniae* or Rd antibodies (<1:10) was comparably protective.

TABLE III. EFFECT OF PRETREATMENT OF SWISS MICE WITH RABBIT ANTISERUM ON MORTALITY FROM *P. mirabilis*.[a]

| | P. mirabilis | | | |
|---|---|---|---|---|
| | $1.5 \times 10^8$ ip | | $5 \times 10^8$ iv | |
| iv Pretreatment (0.25 ml) | (N) | % Mortality | (N) | % Mortality |
| None | (20) | 80 | (25) | 68 |
| Saline | (18) | 78 | | |
| *E. coli* J5 (Rc) antiserum | | | (30) | 27 |
| Pre-immune serum | | | (30) | 27 |
| *S. typhimurium* SL 1032 (Rd) antiserum[b] | (40) | 85 | (32) | 25 |
| Preimmune serum[b] | (16) | 81 | | |
| *S. minnesota* 595 (Re) antiserum | (18) | 83 | (24) | 42 |
| Preimmune serum | (18) | 83 | (25) | 32 |
| *P. mirabilis* antiserum | (18) | 6 | (25) | 4 |
| Preimmune serum | (20) | 80 | (27) | 30 |

[a] Each vertical column gives the results of two combined studies.
[b] Heated to 56°, 30 min.

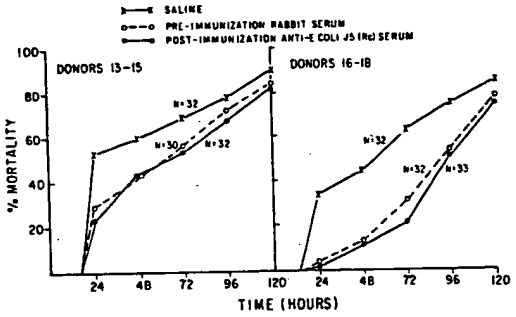


FIG. 2. Effect of i.v. pretreatment of Swiss mice with pooled rabbit antisera (0.25 ml) to *E. coli* J5 (Rc chemotype) on mortality from iv *K. pneumoniae* ($1 \times 10^4$). Sera from donors 16–18 (end panel) heated to 56°C, 30 min.

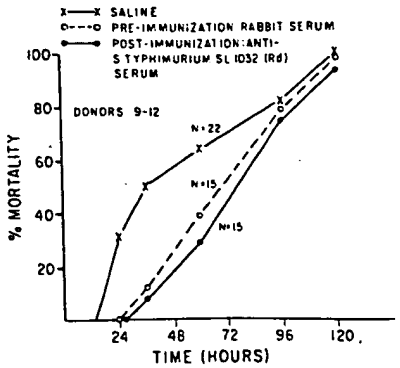


FIG. 3. Effect of i.v. pretreatment of Swiss mice with pooled rabbit antisera (0.25 ml) to *S. typhimurium* SL 1032 (Rd chemotype) on mortality from iv *K. pneumoniae* ($1 \times 10^4$).

Figure 4 demonstrates the ability of rabbit antisera to the Re chemotype of *S. minnesota* 595 mutant to protect mice against iv *K. pneumoniae* challenge. Pretreatment with antisera from 3 different donor sources (2 individual and 1 pool) retarded mortality but preimmune sera from the corresponding donors (panel 1 and 2) or from other normal non-immunized donors (end panel) possessing no detectable anti-*K. pneumoniae* or Re antibody titers (<1:10), provided as effective or greater protection.[3] In contrast to the anti-Re sera, specific antiserum to the *K. pneumoniae*, possessing a *K. pneumoniae* agglutinating titer of 1:640, provided complete protection (end panel). As seen in the end panel of Fig. 4, heating normal or immune rabbit sera to 56° for 30 min did not abolish protective activity.[4]

[3] Pre and post-immune sera from different donors rather than from corresponding donors were employed in studies shown in end panel of Fig. 4 to exclude a role of prebleeding in preventing the appearance of enhanced protective activity in the rough mutant antisera.

[4] For the *K. pneumoniae* studies, it was found essential that rabbits be immunized with rough mutants in areas completely separated from those used for infecting mice. In early studies, immunization of rabbits was carried out two rooms removed from those used for experimental infection. Increases in specific agglutinating antibody titers to *K. pneumoniae* appeared during immunization with the rough mutants (from <1:10 to a final titer of 1:160). Pretreatment of mice with such rough mutant

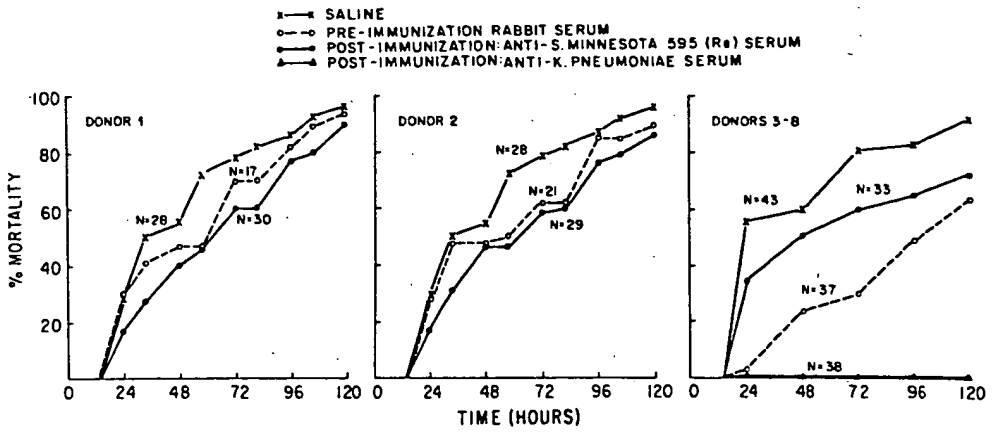


FIG. 4. Effect of i.v. pretreatment of Swiss mice with rabbit antisera (0.25 ml) to *S. minnesota* 595 (Re chemotype) on mortality from iv *K. pneumoniae* ($1 \times 10^4$). Individual animals (donors 1 and 2) supplied both pre and postimmunization sera. Donors 3–8 (end panel) supplied only post-immunization *S. minnesota* 595 antisera; the pre-immunization sera and post-immunization *K. pneumoniae* antisera were obtained from other donors.

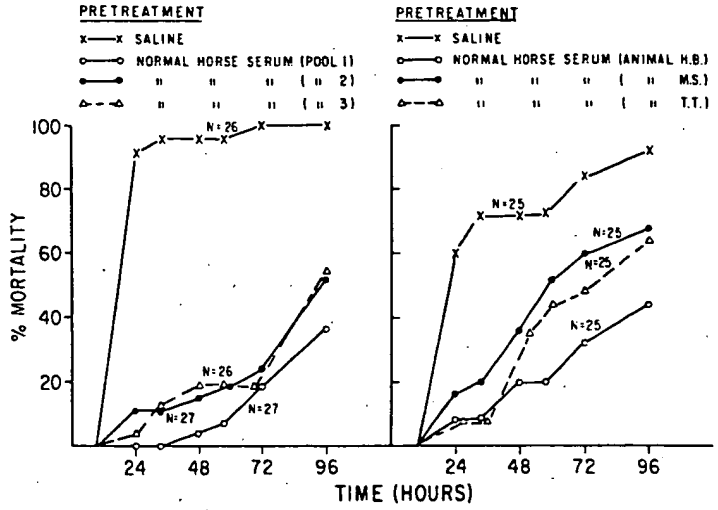


FIG. 5. Effect of iv pretreatment of Swiss mice with normal horse serum (0.25 ml) from 6 different sources on mortality from i.v. *K. pneumoniae* ($1 \times 10^4$).

*Effect of pretreatment with normal horse serum.* Normal horse serum obtained from 6 different sources, all provided unequivocal protection to mice against iv challenge with *K. pneumoniae,* Fig. 5. Pooled horse sera, obtained from three different commercial sources, were from donors reputed to be healthy and never vaccinated with bacterial antigens other than tetanus toxoid. Sera from the individual donors were from privately owned healthy animals known never to have been inoculated with any bacterial vaccine. Despite the protective activity, the normal horse sera possessed no detectable (<1:10) agglutinating antibodies to the *K. pneumoniae* or to the Rd or Re mutants used in the present studies. All of the horse sera possessed anti-Ra (*S. minnesota* R60) agglutination titers of 1:40 and anti-Rc (J5 mutant of *E. coli* 0111) titers ranging from 1:40 to 1:320.

Quantitative studies on the protection con-

antisera conferred significant protection against iv *K. pneumoniae* challenge when compared with the corresponding pre-immune sera. However, these increments in anti-*K. pneumoniae* antibodies and in protective activity were entirely circumvented when a separate building was used for rabbit immunization, and these are the data shown in the above studies.

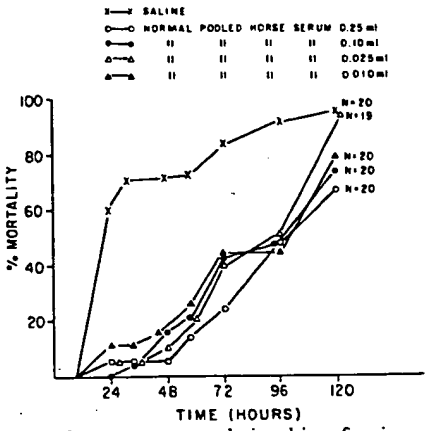


FIG. 6. Dose-response relationships for iv normal horse serum (pool 1 shown in Fig. 5) and protection against iv *K. pneumoniae* ($1 \times 10^4$) in Swiss mice. Horse serum doses less than 0.25 ml were reconstituted with pyrogen-free sterile physiologic saline to 0.25 ml.

ferred by normal horse serum against iv *K. pneumoniae* challenge indicated that as little as 0.01 ml could provide unequivocal protection, Fig. 6. To explore the nature of this protection, aliquots of normal horse serum were absorbed with the challenge strain of *K. pneumoniae* or with a heterologous smooth gram-negative organism (*P. mirabilis*). Absorption was performed using a 1% suspension of live organisms previously grown overnight in trypticase soy broth and washed 3 times in pyrogen-free sterile saline. Four absorptions were carried out for 15 min each at 0°, and the serum then sterilized by passage through a Millipore filter; control nonabsorbed serum aliquots were also passaged through a Millipore filter. The absorbed sera were shown to be sterile before testing for protective activity. As seen in Fig. 7, protective activity of normal horse serum was clearly reduced by absorption with *K. pneumoniae* but not with *P. mirabilis*. As estimated from the dose-response protection afforded by this batch of normal horse serum (Fig. 6), a high proportion (>95%) of the protective activity was specifically removed by absorption with *K. pneumoniae*. No decline in the anti-Ra or Rc titers (1:40 and 1:80 respectively) was detected in this absorbed serum. In contrast to horse serum, protective activity of normal rabbit serum against iv *K. pneumoniae* challenge was not reduced by comparable absorption with viable *K. pneumoniae* (2 separate trials).

*Discussion.* In 1968, Chedid and coworkers reported that antisera from horses hyperimmunized with an Ra chemotype of a rough *Salmonella* mutant (*S. typhimurium* TV 119) protected mice against iv challenge with *K. pneumoniae* (1). Subsequently, McCabe reported that antisera from rabbits immunized with an $Rd_2$ or Re chemotype of rough *Salmonella* mutants (*S. minnesota* $Rd_23$ and Re 595), but not with the Ra (or Rb, Rc, or $Rd_1$) chemotypes, protected mice against iv *K. pneumoniae* or *E. coli* challenge (2). Young and coworkers later reported that rabbit antiserum to the Re chemotype of *S. minnesota* 595 protected mice against ip challenge with *E. coli* (3). In the above studies, each investigator concluded that the broad spectrum protection conferred by pretreatment with the rough mutant antisera was mediated by antibodies to core antigens common to the *Enterobacteriaceae*. These core antigens were presumably "unmasked" in the rough mutants, allowing production of high titers of cross-reactive protective antibodies. Recently, Ng and coworkers repeated these studies with rabbit antisera prepared against the same Re chemotype of the *S. minnesota* 595 mutant used by the latter two groups of investigators, as well as with antisera to an Re chemotype of an *S. typhimurium* mutant (SL 1102). While increments in antibodies to common core antigens were clearly demonstrated, pretreatment of mice with either of these antisera afforded no protection against iv challenge with *S. typhosa, E. coli, P. aeruginosa,* or *K. pneumoniae*; the latter organism was the identical challenge strain employed by the initial two groups of investigators (4).

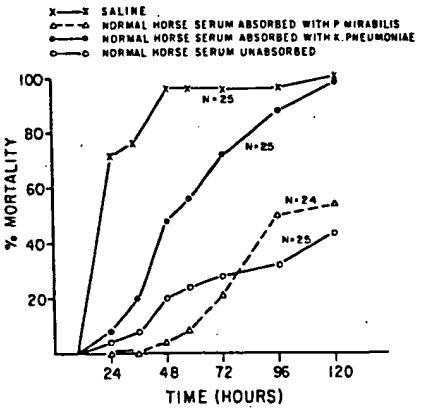


FIG. 7. Effect of absorption of normal horse serum (pool 1 shown in Figs. 5 and 6) with viable *K. pneumoniae* or *P. mirabilis* on protective activity against iv *K. pneumoniae* ($1 \times 10^4$) in Swiss mice. 0.25 ml horse sera were given iv as pretreatment in all trials.

This inability to confirm previous observations could not be readily explained, although it paralleled earlier observations by Mullan and coworkers and by McCabe and Greely that rabbit antisera to other common antigens of *Enterobacteriaceae*, i.e. lipid A and common enterobacterial antigen, failed to protect mice against parenteral infection with smooth *Enterobacteriaceae* (5, 6). The present findings are in agreement with those of Ng and coworkers. Rabbit antisera prepared against 3 different rough *Enterobacteriaceae* mutants, an Rc, Rd, and Re chemotype of *E. coli, S. typhimurium,* and *S. minnesota* respectively, although containing high titers of agglutinating antibodies to the mutant organisms, provided no more protection to mice against challenge with 3 different species of smooth *Enterobacteriaceae* than did the preimmune serum from the same donors lacking any detectable antibodies to the rough mutants. To minimize the possibility that these negative results might be secondary to heterologous serum toxicity (some fresh rabbit sera were acutely lethal for mice), heat-inactivated sera (56°, 30 min), found in our laboratory to possess reduced toxicity for mice, were also tested. The results remained unaltered. It is emphasized that challenge with each bacterial species, *E. coli* 018, *P. mirabilis,* and *K. pneumoniae,* was performed with inocula carefully titrated to produce mortalities within the sensitive dose-response range and that bacterial replication appeared necessary for lethality in these models since heat-killed inocula produced no mortality. Moreover, these findings with the rough mutant antisera contrasted markedly with specific antisera which consistently conferred high levels of protection against the respective challenge organisms, significantly greater than that conferred by preimmune serum from the corresponding donors.

One possible explanation for our and Ng and coworkers inability to confirm the conclusions of previous investigators became apparent from the present studies. The preimmune normal rabbit and horse sera tested in the present studies conferred definite protection to mice against each of the 3 smooth challenge organisms, including *K. pneumoniae,* type II, the identical strain employed by the previous investigators. Analysis of sera from individual normal donors revealed a wide spectrum of protective activity. Because of this variable protective activity of normal serum, it was found necessary that sera be obtained prior to immunization, stored frozen, and tested concomitantly with immune sera from the corresponding donors (both pooled in the same proportions) in order to determine whether the immune sera were protective by virtue of an increase in antibody content. Thus, whereas Chedid and co-workers observed that sera from horses hyperimmunized with the Ra mutant *S. typhimurium* TV 119 protected Swiss mice against *K. pneumoniae,* the corresponding normal pre-immune sera were not tested. (Indeed, no normal horse sera were tested). The present findings indicate that normal horse serum from non-immunized healthy animals can impressively protect Swiss mice against *K. pneumoniae* infection. Moreover, this prophylactic activity could be reduced markedly (>95%) by absorption of normal horse serum with the homologous smooth *K. pneumoniae,* but not with a heterologous smooth gram-negative bacterial species (*P. mirabilis*), suggesting that natural specific antibodies account for the major portion of such protective activity. This is supported by the finding that while all the normal horse sera possessed agglutinating antibodies against Ra and Rc (but not Re) chemotypes of *Enterobacteriaceae* the loss of protective activity following absorption with *K. pneumoniae* was unaccompanied by any decline in the anti-Ra or Rc titers. It is of interest in this regards that Chedid and coworkers similarly observed that absorption of their horse *S. typhimurium* TV 119 antiserum with smooth *K. pneumoniae,* but not with other smooth gram-negative bacteria, very effectively reduced its protective activity against *K. pneumoniae* (1). We cannot readily explain the discrepancy between our findings and those of McCabe (2). At least one of the challenge organisms employed in both studies, *K. pneumoniae,* type II, was the identical strain, and hemagglutinating antibodies to the Re mutant *S. minnesota* 595 in the rabbit antisera were equally high. Differences in mouse strains might explain the variance in results (CF1 mice were employed by McCabe). However, it appears that pre-immune sera from the same donors were not employed for control purposes in the previous studies and this might account for the protection by

antiserum appearing causally related to immunization with the $Rd_2$ and Re *Salmonella* mutants. We also cannot readily explain the discrepancy between our findings and those of Young and co-workers who reported that heat-inactivated rabbit antiserum to the Re mutant of *S. minnesota* 595 given ip to mice yielded significantly greater protection against ip *E. coli* challenge than did heat-inactivated normal rabbit serum (3). Possibly, the fact that different *E. coli* and mouse strains were employed by Young and co-workers (i.e., *E. coli* 085HA and CD mice) or that hog gastric mucin was used to enhance infection in these previous studies could account for the divergent conclusions.[5] On the other hand, it is again emphasized that pre-immune sera from the same rabbit donors apparently were not used for control purposes. If the variable protective activity of normal rabbit serum observed in the present studies extends to other *E. coli* strains, the need to use preimmune sera from the same donors as controls for antisera protection becomes crucial. This reasoning is supported by the data of Hodgin and Drews (8) wherein normal rabbit sera significantly protected NMRI mice against i.p. challenge with *E. coli* 04 when compared with physiologic saline ($P < 0.01$ for normal serum given ip and $P < 0.05$ for normal serum given iv). In contrast, no significant difference is evident when the protective activity of antiserum to the *S. minnesota* 595 Re chemotype is compared with normal rabbit serum ($P \sim 0.2$).

The ability of normal rabbit serum to confer broad spectrum protection against gram-negative bacterial infection in mice of various strains has been noted previously (4, 5, 8, 9). The present studies confirm and extend these observations to include normal horse serum. Although the mechanism underlying such protection was not studied, it may differ depending upon the donor species. For example, in the present studies, absorption with *K. pneumoniae* sharply reduced the protective activity of normal horse serum against *K. pneumoniae* infection, but did not do so with normal rabbit serum. Galanos and co-workers have suggested that complement might be responsible for the protective activity of normal rabbit serum in mice, at least with respect to *S. typhimurium* infection (9). Since heat-inactivation of normal rabbit serum did not abolish its protective activity against the 3 *Enterobacteriaceae* used in the present studies, if rabbit complement is responsible for protection in these models it would be the heat stable components. These might act in conjunction with the murine complement system, possibly by enhancing the characteristically deficient bactericidal activity of mouse serum (10, 11). In any case, this protective activity of normal serum raises the possibility that the present inability to detect enhanced protection by rough mutant antisera might be related to a "masking" effect of the already existing normal protective system. This appears unlikely, however, since in those instances where minimal or no protection was conferred by the pre-immune sera (donors 1 and 2, Fig. 4; ip challenge, Table III) no enhanced protection was seen with the corresponding rough mutant antisera. The above considerations emphasize the need to use pre-immune serum from the same donors for control purposes when studying the protective activity of heterologous antisera to rough gram-negative bacterial mutants in murine models. When this is done, the conclusion that antisera to rough gram-negative mutants confer broad spectrum protection to mice against parenteral challenge with smooth *Enterobacteriaceae* because of the rise in antibody titer to common core antigens cannot be supported.

*Summary.* Rabbit antisera to three rough *Enterobacteriaceae* mutants, the Rc chemotype of *Escherichia coli* J5, the Rd chemotype of *Salmonella typhimurium* SL 1032, and the Re chemotype of *Salmonella minnesota* 595, were administered iv or ip to outbred Swiss albino mice. Control animals were injected concomitantly with normal serum from the same donors obtained prior to immunization. One hour later, challenge was performed ip or iv with $LD_{95-100}$ doses of viable *Escherichia coli* 018, *Proteus mirabilis*, or *Klebsiella pneumoniae*. Normal pre-immune rabbit sera, lacking detectable antibodies to the specific challenge bacterial strains or to Ra, Rc, Rd, or Re rough mutants of *Enterobacteriaceae*,

[5] Young and coworkers have subsequently reported that the protection conferred by rabbit antiserum to the Re *S. minnesota* 595 mutant against i.p. *E. coli* challenge was "critically dependent" upon the concentration of hog mucin injected (7).

exhibited definite abilities to reduce septic mortality when compared with physiologic sterile saline. Analysis of preimmune sera from individual rabbit donors revealed a wide spectrum of protective activity. Post-immune sera against the rough bacterial mutants, possessing high titers of Rc, Rd, or Re antibodies, conferred no protection above that afforded by the corresponding preimmune sera. Only antisera to the specific challenge bacterial strain proved more protective than the corresponding pre-immune sera.

Normal horse sera from 6 different sources, obtained from healthy animals never immunized with gram-negative bacterial vaccines, all possessed agglutinating antibodies against Ra and Rc (but not Re) chemotypes of *Enterobacteriaceae* and all provided high levels of protection aginst iv *K. pneumoniae* sepsis in mice. However, naturally occurring specific antibodies, not the anti-rough mutant antibodies, appeared primarily responsible for such protection since protective activity was markedly reduced (>95%) by absorption with the homologous (*K. pneumoniae*) but not with a heterologous (*P. mirabilis*) smooth species and since this loss of protective activity was unaccompanied by any decline in anti-Ra or Rc titers.

The findings fail to support the conclusion that antisera to rough gram-negative bacterial mutants confer broad spectrum protection to mice against parenteral challenge with smooth *Enterobacteriaceae* because of the rise in antibody titer to common core antigens.

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